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The overall goal of the studies in our proposal is to establish the feasibility of delivering corrective genes to chronically wounded skin, using JEB keratinocytes as an *in vitro* model system, and address the issues of sustained targeted temporal and cell-type specific expression. The next step in the transfer of this technology to chronically wounded skin will be the application of *ex vivo* gene delivery by grafting of genetically engineered keratinocytes. The data produced in this study will provide results that demonstrate the safety, efficacy and feasibility of a genetic therapy for wound healing using JEB as a cell culture model system. It is anticipated that these results may be directly relevant to the development of novel wound-care treatment modalities which synthetically recapitulate basement membrane components, particularly in light of the recent success demonstrated by enhanced wound healing using genetically-engineered "bandages" of keratinocytes expressing genes of interest.

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Table of Contents

4. Introduction

Following the events of September 11, 2001, the military relevance of our work suddenly seemed that much more significant, and therefore, much of our progress in the second award period was driven by our desire **to focus on the development of a universally applicable skin equivalent that could be easily manufactured and applied without skilled surgical experts in grafting techniques or the need for gene introduction using exogenous means.** Chemical warfare agents such as sulfur mustard (bis [2-chloroethyl] sulfide) are potent cutaneous vesicants which cause frank blistering in the skin by inducing separation at the dermal-epidermal junction. At the ultrastructural and histological level, the blisters characteristic of hemidesmosomal subtypes of junctional EB are quite similar to those induced by chemical vesicants such as sulphur mustard. Although the mechanisms of vesicant-induced blistering is not well understood, several studies in the literature point to structural components in the basement membrane as potential targets for vesication. Several of these targets, including laminins and BPAG2, are the same protein components which are missing or abnormal in patients with the hemidesmosomal subtypes of JEB. Therefore, using JEB cells as an *in vitro* model system, we initially developed the methodology for replacement of these proteins by gene therapy into deficient cells. **However, it became clear in the course of our work that we need not necessarily focus on one particular gene over another, but rather on the development of a universally acceptable source of epithelial cells that are**

genetically intact and not subject to immune rejection between donors and recipients.

The ability to create genetically enhanced skin grafts has direct implications toward the improvement of combat readiness and increased rates of wound healing in military situations in which chemical warfare agents have been utilized. The feasibility of grafting genetically-modified keratinocytes for enhanced wound healing has recently been demonstrated using an agent which increases neovascularization in the wound bed. Keratinocyte grafts could serve as a temporary, readily available, and easy to apply allograft in military situations when the initial 7-14 day window for wound healing is critical. This type of **cell-based method** for the synthesis and delivery of local basement membrane zone components could rapidly provide the initial scaffold essential for re-epithelialization during wound healing. **Development of such a technology would thereby greatly speed the initial wound healing process, and return military personnel injured with cutaneous vesicants to combat-ready status faster and with an improved rate of wound closure.**

Overall Goals and Hypothesis

The overall goal of our studies was to establish the feasibility of delivering cellular 'bandages' to chronically wounded skin, using JEB keratinocytes as an *in vitro* model system, and addressed the issues of

sustained targeted temporal and cell-type specific expression. The next step in the transfer of this technology to chronically wounded skin will be the application of *ex vivo* gene delivery by grafting of genetically engineered keratinocytes. **It is anticipated that these results may be directly relevant to the development of novel wound-care treatment modalities which synthetically recapitulate basement membrane components, by enhanced wound healing using genetically-engineered "bandages" of keratinocytes.**

The overall goal of the studies in our proposal was originally to establish the feasibility of delivering corrective genes to chronically wounded skin, using JEB keratinocytes as an *in vitro* model system, and to address the issues of sustained targeted temporal and cell-type specific expression. The next step in the transfer of this technology to chronically wounded skin will be the application of *ex vivo* gene delivery by grafting of genetically engineered keratinocytes. The data produced in this study will provide results that demonstrate the safety, efficacy and feasibility of a genetic therapy for wound healing using JEB as a cell culture model system. It is anticipated that these results may be directly relevant to the development of novel wound-care treatment modalities which synthetically recapitulate basement membrane components, particularly in light of the recent success demonstrated by enhanced wound healing using genetically-engineered "bandages" of keratinocytes expressing genes of interest.

During the second period of the project we continued to build and evaluate the experimental model. Specifically, we have established the recombinant model skin equivalents for the *in vitro* gene therapy experiments. We have been focusing on creating the model systems to be used for the *in vitro* gene therapy experiments. The critical elements of this system are 1) the gene construct which is now completed, 2) the gene delivery methodology, and 3) the *in vitro* skin model.

5. Body

I. Progress in Task 1: To create a recombinant plasmid vector containing the entire coding sequence of BPAG2 (completed)

a. Rationale, Approaches and Design

As detailed in the first progress report, we devised a detailed gene assembly strategy for BPAG2 and successfully proceeded with the construction of the therapeutic gene construct, mainly relying on PCR and traditional cloning techniques. During the design preparation, we took into consideration the different requirements of the potential delivery techniques as well, and included a FLAG tag to facilitate immunodetection. Task I was essentially completed during the first 12 month period.

II. Progress in Task 2: To transiently express the BPAG2 cDNA and monitor its expression in vitro (18-30 months)

a. Rationale and Experimental Design

As a preparation for gene replacement experiments, during the first award period, we had already begun to test and compar the available delivery techniques by performing a series of transfection experiments using reporter gene systems (such as GFP) or antibiotic selection to assess efficiency. During these experiments, we used keratinocytes and compared different non-viral methods of delivery, including cationic liposomes and macromolecules, and focused on lipofectamine as the method of choice. Using these systems, we were able to accomplish high efficiency (of approximately 15-25% of cells) and long term gene expression (2-3 weeks).

To further optimize our gene-delivery techniques during the second award period, after completing the *in vitro* gene delivery experiments in the first phase of this project, we performed *in vivo* gene-delivery experiments, to compare and evaluate the available methods. This approach was selected because the literature suggests that the characteristics of the living skin resemble the characteristics of the *in vitro* skin model more closely than that of the cells of a monolayer culture.

We have devised successful delivery strategies and were able to efficiently deliver different DNA constructs to mouse skin using topical application of liposome encapsulated DNAs as well as non-viral techniques. To evaluate our delivery methods we used a model gene system different from those that we will use in the actual experiments of this project, simply using GFP-labelled oligonucleotides (Cserhalmi-Friedman et al, 2003). The use of a separate model system was necessary to simplify the evaluation of the efficiency of the delivery. These *in vivo* experiment allowed us to devise a delivery strategy that we would be able to use in the *in vitro* gene therapy experiments of this project, but also in the subsequent *in vivo* model based project.

b. Outcomes, Results and Future Directions

An alternative nonviral method we had considered for the introduction of exogenous genes into cultured keratinocytes is by bombardment with DNA-coated gold particles using a particle accelerator (BioRad) or gene gun. After particle bombardment, many cultured cells will express the transgene, and a few may contain stable integrated copies. The gene gun has also been used to modify skin *in vivo,* with about 20% of epidermal cells expressing the transgene (Yang, 1992). Similar to our experience, however, expression was lost in 2-3 weeks, suggesting that this method of introduction was no more effective and far more complicated that simple transfection with Lipofectamine. Thus, our success

in gene transfection into keratinocytes obviated the need to engage this technology.

III. <u>Progress in Task 3:</u> To transfect the BPAG2 cDNA into cultured GABEB patient keratinocytes and monitor their expression in recombinant skin models (18-36 months)

<u>a. Rationale, Approaches and Design</u>

We have been working most extensively and focusing much of our efforts on the *in vitro* skin model described in the proposal. We have initiated extended cultures of keratinocytes and fibroblasts used in the assembly of the final skin model. To be able to study the characteristics of the proposed model, we first proceeded with the assembly of a model consisting of normal cells, to be used as a control during the recombination experiments. According to the experimental outline, we performed a two-step assembly. During the first phase, we developed a multi-layer fibroblast base, which served as a surprisingly fertile acceptor surface for the keratinocytes which were applied n the second phase. After successful attachment of the seeded keratinocytes, the recombinant system was elevated to the air-liquid interface to allow the multi-layer growth and differentiation of the keratinocytes. We have been most fortunate to recruit the expert advice of Dr. Rebecca Morris in the Department of Dermatology to assist

us with this work. Dr. Morris is one of the founders of the keratinocyte stem cell field and has vast experience with recombinant skin models and particularly inducing differentiation in culture systems.

To accomplish the goal of creating a cellular bandage to facilitate wound healing, we created the *in vitro* skin model described in the proposal. We built the skin models using different combinations of starting materials. All culture systems were based on a collagen matrix, which contained a variable proportion of fibroblasts. These dermal equivalents could then be covered with either wild-type or EB keratinocytes. Their attachment, cell division and growth characteristics were observed and evaluated. The human cell based models were compared side-by-side with skin models utilizing cell types of murine origin and different combinations thereof.

b. Outcomes, Results and Future Directions

During the second period, we fully mastered with the technologies of skin equivalents and determined the optimal density of cell seeding, times in culture, differentiation conditions and requirements to achieve epidermal differentiaion in culture. Moreover, we gained a growing appreciation for the observation by ourselves and others that keratinocytes need not necessarily be genetically modified in order to perform well in our assays. In fact, control keratinocytes simply expressing normal (and not excess) amounts of BPAG2 displayed growth

characteristics equivalent with those that had been transfected. This led us to hypothesize that perhaps our approach could once again be refined by the data gathered in year 1 and 2, and going into year 3, **we sought to identify a population of normal keratinocytes (or potentially, another source of epithelial cells) that could serve as universal donor cells in universally applicable bandages.**

6. Key research accomplishments

During the second award period, our main accomplishments revolved around continued evaluation of the *in vitro* model system, and in particular, we focused intently on the development of the recombinant skin models. We succeeded in refining the culture conditions and cellular compositions of recombinant skin composed of fibroblasts and keratinocytes, and were able to induce differentiation by lifting to the air interface. These achievement bode well for our goal in year 3 of developing a simple, universally applicable bandage for use in chemical burns as well as EB. Furthermore, during year 2, we began to

investigate the potential of using alternative sources of epithelia in our graft systems in place of keratinocytes. Kindly refer to Section 8 below (Conclusions) for more details regarding our future directions after the second award period.

7. Reportable Outcomes

Publications 2000-2001 in Year 2

1. Cserhalmi-Friedman, P., Anyane-Yeboa, K. and Christiano, A.M. DNA based molecular analysis in the rapid diagnosis of Herlitz Junctional EB. (2001) Clin. & Exp. Dermatol. 26:205-207.

2. Cserhalmi-Friedman, P.B., Olson, P., Champliaud, M-F., Brunken, W., Burgeson, R.E. and Christiano, A.M. (2001) Structural Analysis of the LAMC3 Gene. Biochem. Biophys. Res. Comm. 280:39-44.

3. Cserhalmi-Friedman, P.B., Garzon, M.C., Guzman, E., Martinez-Mir, A., Yeboa, K. and Christiano, A.M. (2001) Maternal Germline Mosaicism in Dominant Dystrophic Epidermolysis Bullosa. J. Invest. Dermatol. 117:1327-1328.

4. Cserhalmi-Friedman, P.B., Yeboa, K. and Christiano, A.M. Paternal Germline Mosaicism in Herlitz Junctional Epidermolysis Bullosa. Exp. Dermatol. (in press).

5. Fine, J-D., Eady, R.A.J., Bauer, E.A., Briggaman, R.A., Bruckner-Tuderman, L., Christiano, A.M., Heagerty, A., Hintner, H., Jonkman, M., McGrath, J.A., McGuire, J., Moshell, A., Shimizu, H., Tadini, G. and Uitto, J. (2000) Revised Classification System for Inherited Epidermolysis Bullosa: Report of the Second International Consensus Meeting on Diagnosis and Classification of Epidermolysis Bullosa. J. Amer. Acad. Dermatol. 42:1051-1066.

6. O'Shaugnessy R.F.L. and Christiano, A.M. (2001) Stem Cells in the Epidermis. Skin Pharmacol Appl Skin Physiol. 14:350-357.

8. Conclusions and Future Directions

This period of the research project was highly successful in evaluating and completing the systems and techniques that will be utilized to perform the in vitro experiments aimed at skin gene therapy. During the second award period (2000-2001), like the first, several papers began appearing in the literature that once again helped us to **re-evaluate the status of our work and continually challenge ourselves to be sure we were staying at the forefront of this field, and at the same time, fulfilling the overall goals of the grant, in particular, therapies for EB and chemically-induced blistering.** Clearly, this project has evolved rapidly from where it began as proposed 4-5 years ago, with simple introduction of one gene into keratinocytes, which at the time seemed a

formidable goal, but has since been achieved (Seitz et al, 1999). However, with the field advancing equally rapidly, it was incumbent upon us to modify and adapt our approach accordingly in order to capitalize on the extraordinary progress in other fields in the first 24 months of this grant (1999-2001). **In particular, the advances in stem cell biology, tissue rejection, and organ engineering all came into play for us as we contemplated the development of a universal donor skin.**

The concept of 'stem cell plasticity' emerged fully during 2000-2001, and it became clear that our previous notion of the potential of a stem cell was far too limited. We learned that multi-potent progenitor cells resided in almost every tissue, and that these could potentially be coaxed down various differentiation pathways depending on the conditions of culture. Importantly, many groups began to focus on the skin as a source of donor stem cells that could be transformed into brain, muscle and other tissues (Toma et al, 2001). Drawing on this important body of literature, we instead asked the opposite question – could other epithelia likewise potentially be coaxed into becoming skin? **This question formed the basis for our direction in the final year of this award, and we believe has brought us closer conceptually to the potential to create a universally acceptable skin equivalent for use in military situations.**

9. References

Please see above.

Cserhalmi-Friedman, P.B., Dietz, H.C., Christiano, A.M. Novel Methodology of Use of GFP Constructs to Test Catalytic Oligonucleotides. Exp. Dermatol. (in press).

Seitz, C.S., Giudice, G.J., Balding, S.D., Marinkovich, M.P. and Khavari, P.A. BP180 Gene delivery in junctional epidermolysis bullosa. Gene Ther. 6:42-47, 1999.

Toma, J.G., Akhavan, M., Fernandes, K.J., Barnabe-Heider, F., Sadikot, A., Kaplan, D.R., Miller, F.D. Isolation of multipotent adult stem cells from the dermis of mammalian skin. Nat Cell Biol. 3:778-84, 2001.

Yang, N.S. Gene transfer into mammalian somatic cells *in vivo*. Crit. Rev. Biotech.12:335-356, 1992.

10. Appendices

Publications #2 and 6 are included as representative examples.

Biochemical and Biophysical Research Communications 280, 39–44 (2001)
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Structural Analysis and Mutation Detection Strategy for the Human LamC3 Gene

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Laminins are heterotrimeric extracellular matrix molecules, present in a wide range of basement membranes within human tissues. They consist of a combination of different α, β, and γ subunits. Three different γ subunits have been described to date. Two of them, the γ1 and γ2 chains are constituents of basement membrane related laminins, while the γ3 chain was detected in skin, heart, lung, reproductive tract, brain, and in the retina. Unlike other laminins, the expression of the γ3 chain was localized to peripheral nerves and to the apical surface of ciliated epithelial cells and in the retina. To further investigate the function and the possible pathogenic role of laminin γ3 in human disease, we elucidated the structure of the corresponding LAMC3 gene which encodes this polypeptide. Here we report the genomic organization of the LAMC3 gene and a mutation detection strategy for use in genetic studies. © 2001 Academic Press

Laminins are heterotrimeric molecules consisting of three polypeptide subunits which are found in a wide variety of tissues, mainly as structural proteins, but also playing a role in signal transduction. The different characteristics and functions of laminin molecules depend on the combination of subunit components. Several different polypeptide subunits have been identified to date. They are distributed into subgroups of α-chains, β-chains, and γ-chains, while the genes encoding these polypeptides are designated LAMA, LAMB, and LAMC genes respectively (1). The molecules have an amino-terminal short arm and a carboxyl-terminal long arm, where the short arm consists of globular domains separated by epidermal growth factor like domains and the long arm consists of heptad repeats (2). Their structural and signal transduction functions are thought to play an important role in the formation and maintenance of basement membranes and their interactions with neighboring structures (3).

A total of three laminin γ-chains have been identified, including the γ1 (LAMC1), γ2 (LAMC2), and γ3 chains (LAMC3). Two of them, the γ1 and γ2 chains, are components of laminin 4 and laminin 5, which comprise important structural components of the basement membrane zone of epithelial tissues (4). The cDNA encoding the γ3 chain was recently described (5). Although this laminin gene contains structurally homologous domains similar to laminin γ1, it seems to play a different role in the body by virtue of distinct expression pattern. While the γ3 chain is present in a number of different tissues, such as skin, eye, heart, lung, reproductive tract and placenta, its localization within the tissues is unusual (Koch *et al.*, 1999; Libby *et al.*, 1999). In the cutaneous basement membrane, γ3 is detectable only at nerve crossings and surrounding the Merkel cells in whisker follicles. In the tissues of the reproductive tract, γ3 was detectable at the base of the cilia on the apical surface of epithelial cells in monkey, bovine and rat (Koch *et al.*, 1999). Examination of rat bronchial epithelia gave similar results, showing that γ3 was detected at the apical surface of the epithelial cells (Koch *et al.*, 1999). Laminin γ3 is expressed in the neural retina at the apical surface of the retina and in the outer synaptic layer (Libby *et al.*, 1999). Two different γ3 containing laminin molecules have also been isolated from the cerebellum, supporting the hypothesis that laminin γ3 is an important component of the central nervous system (Champliaud, Brunken, and Burgeson, unpublished observation). To further investigate the possible role of LAMC3 via elucidating its potential role in diseases, we have elucidated the exon-intron structure of this gene and developed a mutation screening strategy.

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TABLE 1

PCR Primers to Amplify the Coding Sequence of LAMC3

Exon	AT	Forward primer	Reverse primer
E1	55	GCCCCGGCCAGAGCGCG	GTGCCCCCAGCCCGCGC
E2	55	GGGCATTGTCCTTTCCAGTG	GACCCTCCTACTGCCGTGAC
E3	55	ATGGCACCCTCTCCCCTCTG	CACCCCACAGCAGCCACCTT
E4	55	CCTCCTCTGTGTCTTTTCCC	CTCCCCTTCTGCTAGTCCTT
E5–6	55	ACCATGCTGTTGCCCTTCTC	AGACAGGAAGGGATGGGGAG
E7	55	TGTTGCCATTCTGAATTTGC	GCTTTGGTGCCTCATCAGTG
E8	55	CCCCTTCCTGGCTGATTCAC	CCATCATCTCACCCCCGACC
E9	55	ACTGATCGGGGGGTGTCTGT	CAGCAGGAGGAACCGACACA
E10	55	GATTTTGATTTCCTACCCAC	CTCAGACAGAAGGGGATACT
E11	55	GGGATCTCACTTTGACCGCT	GTCTTGGGCATGTGGTTGTC
E12	55	GCTTGTGCCTCCTCTCTTCC	TACTCATCCCGCTGTTTTGG
E13	57	TGCTGCCTCTGCCTGGG	CCCCGGGCAGGAGTCAT
E14	61	TTCACACCCACCCTCATCCC	CCAGGGCAGAAGAGAAGGGG
E15	55	TCCCAACACCCCTTCCTGAT	CCCGATGCCTTCTTCCCTAC
E16	55	CCTCAGACCCAGTTCCTTCC	CCATCAGTCATCTCTGCCCA
E17	55	CTTAGGGCATTCTGGGAACA	CTGGCACTTGCTGGGAATAC
E18	61	AGCGGGAGTGTCTGGGCAGG	CTCCCCTTGCCTCCCACCCT
E19	55	TGCCTTTGGGACTACCTGTT	AACATCCTCTCGCTGAATCC
E20	55	CTCCGCTCACTGAATGCCGA	GGCACCTGTGGGGCTGACTC
E21	55	AACAGAAACAGGGCATGGAG	GAACGTCAGGACTCAAGGGA
E22	61	GACAGGGATGGGGAGGGCTA	CGACCCCACACGGAGNTNTG
E23	61	GGNTTGGGTTCTTGGCTGGC	CCTCCTGCAACCCTGACCCT
E24	55	ATTTGGTGATGATTTGGGCA	GGGCGTTCGTCATTACACAG
E25	55	TTCAGACCTCCACTCGTTGT	GGAGTAGTCAGCACGGATGT
E26	51	TTCTTCTCCCTGCCACTGCC	CAGGGGCAGGAGGCGATAGG
E27	55	CCCTACCCTATCGCCTCCTG	GGCAATGAGGACAAGGAGGA
E28	63	AGGCTNCAGGGGCTGGGAGG	CAGGTGGGGGAGTGTGTGCC

Note. The table contains the PCR primer sets used to amplify the coding sequence of the LAMC3 gene. AT indicates the annealing temperature used for the primer set. While one primer set was usually used to amplify one exon and the flanking intron sequences, because of the small size of intron 5 we amplified exons 5 and 6 together in one PCR reaction.

MATERIALS AND METHODS

Determination of Exon-Intron Borders of LAMC3

To identify the exon-intron boundaries of the LAMC3 gene, oligonucleotide primer pairs were designed based on the cDNA sequence, in locations based on the exon-intron organizations of LAMC1 and LAMC2. The forward and reverse primers were placed at a distance of 50–100 bp from the presumed exon borders. Each primer pair was optimized using the program Oligo Primer Analysis Software (National Biosciences, Inc., Version 4.0). The oligomer primers were 20 nucleotides in length, contained minimal secondary structure, between 45 and 65% G/C content, and were free of the potential for primer-dimer formation. Primers were synthesized using an automated oligonucleotide synthesizer (Applied Biosystems).

For PCR amplification, ~500 ng of human genomic DNA was used as a template, and the amplification conditions were: 96°C for 10 min, followed by 95°C for 45 s; annealing for 45 s; 72°C for 1 min, for 40 cycles, in an OmniGene thermal cycler (Hybaid, Inc.). Alternatively, in some experiments we used bacteriophage P1 DNA isolated from commercially available clones containing large fragments of the human LAMC3 gene, as a template for PCR amplification (Research Genetics). The annealing temperature was individually determined for each set of primers. Amplification buffer contained 1.5 mM $MgCl_2$ and 2 U of AmpliTaq Gold DNA polymerase (Perkin Elmer), in a total volume of 50 μl. Aliquots of 5 μl were analyzed on 2% agarose gel electrophoresis. Samples that generated a distinct PCR product on agarose gel electrophoresis were directly sequenced in both directions using the ABI Prism 310 automated sequencing system (Applied Biosystems, Inc.). Exon-intron boundaries were determined by comparison of the cDNA and genomic sequences.

Radiation Hybrid Mapping

The Stanford G3 and Genebridge 4 Radiation Hybrid Mapping Panels (Research Genetics, Huntsville, AL) were screened by PCR with primer pairs derived from each end of the gene, according to manufacturer's protocols. The 5′ end of the γ3 gene, corresponding to exon 2, was amplified using the following primer pair: MF128, 5′-GCT TAT GAG ATC ACG TAT GTG AG-3′; MF155, 5′-CTG CAG CCC AGG GCT CTC CTC GAA-3′. The 3′ end of the γ3 gene, corresponding to exon 28, was amplified with the following primers: MF143, 5′-TGG GTC ATA CAC ACA GAC ATG CAC-3′; MF144, 5′-TCC CAG CAG CAG GAG CTG CAG ATC-3′.

Mutation Detection Strategy

Primer design and PCR amplification for mutation detection. For amplification of coding sequences of LAMC3, oligonucleotide primers were designed for each exon on the basis of flanking intronic sequences in order to generate PCR products containing the exon and parts of the adjacent intron sequences. Primer design and synthesis were performed as described above. For PCR amplification, we used ~500 ng genomic DNA, isolated from peripheral blood lymphocytes according to standard techniques (Sambrook *et al.*, 1989). PCR conditions were identical to those described above. Optimal annealing temperature was determined individually for each set of primers (Table 1).

TABLE 2

Exon-Intron Borders of LAMC3

Exon	Splice donor			Splice acceptor
1			CTCCGCCTAG	gtaagcgcgggctggggg
2	gggccccttttcctggctgcag	GGAAGGCTTA	TGGGCTGCAG	gtcagggaggagcggggc
3	caccctctcccctctgccccag	GAGTGGGTCA	TGGGCGGCAG	gtaggagggaggagggag
4	cctcctctgtgtctttttcccag	GTGCAAGTGC	GAGTGTCTGC	gtgagtgtctgagtgtca
5	tttccccactcctgccacatag	CCTGCAACTG	CAGTCGGCAG	gtgagtggactcctgatc
6	ttgccctttcctctccccaaag	GCTCCCTACA	GAGGCTGCAG	gtgatgggcgatggggcg
7	tcttgtcctgtctcattggcag	ACCCTGCACT	TATGTGACAG	gtttgtgagctaatccag
8	gcatgtcctgcctccatttcag	ATGTCGCCCG	TTCCACCAGG	gtaagagatgctccctgc
9	ggtgtctgtgtttgctgtctag	GAGCCGAAGG	ACAGCACCAG	gtacctccagcaccagag
10	agccttcttctcttgtcttcag	GGAAGTTCCT	TCAGGTTCCA	gtaagtatccccttctgt
11	ctttgaccgcttctctcgccag	CCTGCAGGAG	AGCCCTGCCG	gtcagtaaagacaaccac
12	tctctcccttctcgctctgcag	GTCCAGTGTT	CCCAACACAG	gtgagtctcctggcaccc
13	tggtgcttgtcttgcccctcag	GGATCTGTGT	GGCCAGAGAG	gtaatgactcctgcccg
14	cacgtgctccctatacacacag	GGCGGCGCTG	AAATGCATGC	gtgagtacctacctccag
15	cccttcctgatcctgcccccag	CTTGCAGCTG	GCTGCCGGAG	gtaggtagggtgagactg
16	cctgttccttccctgatcacag	CTGCAAGTGT	GGCTGCCGGG	gtaaggaggctgggtcct
17	gcactgcccctggcccctctag	CCTGCAGGTG	GAAGGAGGAG	gtgagtcggccagaccac
18	cttttcttcctgtcctctccag	GCAGCCAAGC	CTGCTTCCAG	gtacagcaggagcgcaga
19	ctgggctgtgggcttcccatag	GGGCTCGGGA	CGCGTCTCTG	gtatcccaggggaccccc
20	cctcttcctcttctttctacag	GAGATTCCTC	TCGCCAGGAG	gtgatgtccaagacatgg
21	caggtgggtgtccgtcacacag	CCACAGAGAC	TGGAGGACAG	gtgaggcctccccaggtg
22	cgtggcctctgcttcctcccag	GTACCAGGAG	GGGAGCTCTG	gtcagctcatttgtctca
23	tgagcagattgtctccctgcag	CCTCAGAAGT	CCTCACAATG	gtcagctcttgtgcttt
24	gcctctcctcatctctctccag	CTGCACCAGG	GATCTGGAAG	gtacgtgagtccagctga
25	ccagcctcagccgggtttgcag	GAATGAAGCT	CAGTGCCAAG	gtcaggggtggtggctgt
26	gccactgccacccatcccatag	CTTGCCAAGG	AGCTGAGCGG	gtacgtttgcagggccct
27	tcttcctcctcccccctggaaag	GTGGGTGCTG	GCCAGGCTGG	gtagggggcctaaggctg
28	ggctgtttgtgccccaccacag	GGTCGCTGGA		

Note. The table demonstrates the exon-intron boundaries in the LAMC3 gene, organized according to exons. The 5′ and 3′ parts of the exon sequences are in capitals.

Heteroduplex Analysis

To test the primer pairs, we used genomic DNA from unrelated individuals from different geographic locations (North America, Eastern Europe, Far East) as a template for PCR amplification. Eight microliters of the PCR products was prepared for heteroduplex analysis using conformation sensitive gel electrophoresis (CSGE). CSGE was performed in a vertical gel containing 10% polyacrylamide (99:1 Acrylamide: 1,4-Bis (acryoyl) piperazine), 15% ethylene glycol, 10% formamide and 0.5× TTE buffer, pH 9.0 (Tris Taurine EDTA buffer, US Biochemicals), with sample preparation consisting of denaturation at 98°C for 8 min followed by a 30 min incubation at 68°C before loading. Heteroduplexes were visualized by staining with ethidium bromide. Bands of altered mobility detected in heteroduplex analysis were directly sequenced using the ABI Prism 310 automated sequencing system (Applied Biosystems, Inc.).

RESULTS

Exon Intron Borders

We determined the complete exon-intron structure of the LAMC3 gene. Comparison of the previously published cDNA with the genomic sequences revealed a total 28 exons (Table 2). The translation initiation codon was present in exon 1, while the translation termination codon was found in exon 28.

Radiation Hybrid Mapping

MF128-155 was linked to marker SHGC-4319 in the Stanford G3 panel, with a lod score of 10.39 and a distance of 0. MF143-144 was linked to marker SHGC-4319 in the Stanford G3 panel, with a lod score of 6.84 and a distance of 17 cR. MF143-144 was linked to marker WI-6494 at a distance of 2.84 cR on the Genebridge 4 Whitehead framework map. In addition, an STS marker derived from the γ-3 sequence was found in the GeneMap98 database. The STS marker WI-14302 (GenBank Accession No. G22528) is derived from an EST which represents the 3′-most exon of the LAMC3 gene. Based upon comparisons of the SHGC and WI RH maps, the LAMC3 gene is probably organized with the promoter toward the telomere and the 3′ end towards the centromere of chormosome 9 at 9q31-34.

Mutation Detection Strategy

Screening of PCR products from unrelated, unaffected individuals from different geographic locations resulted in the detection of several bands of altered mobility. Sequencing the corresponding PCR products,

TABLE 3

Sequence Variants Detected in the Coding Sequence of LAMC3

Exon	Nucleotide	Amino acid
3	789 G-to-C	Ser-to-Thr
3	83 G-to-A	Val-to-Val
4	952 A-to-G	Ala-to-Ala
6	1315 A-to-G	Thr-to-Thr
9	1661 T-to-G	Ser-to-Ala
10	1728 G-to-A	Gly-to-Glu
11	2029 C-to-T	Ser-to-Ser
14	2656 C-to-T	Ser-to-Ser
19	3341 G-to-A	Gly-to-Ser
21	3604 C-to-T	Thr-to-Thr

Note. The table lists the sequence variants we detected in the LAMC3 gene while screening clinically unaffected, unrelated individuals. The first column shows number of the exon, in which the variant was found, the second column shows the nucleotide change according to the published cDNA sequence of LAMC3 (GenBank Accession No. NM_006059, (5)). The third column demonstrates the result of the sequence variant at the protein level.

we were able to demonstrate the existence of different sequence variants. We found single nucleotide polymorphisms that did not result in an amino acid changes in exons 3, 4, 6, 10, 11, 14, 21. We found single nucleotide polymorphisms the did result in single amino acid changes in exons 3, 9, 19 (Table 3). In addition to the sequence variants detected in the coding sequence of LAMC3, we found one variant in a noncoding region. The 3′ region of intron 16 consisted of tandem 31 bp repeats, immediately preceding the last nucleotide of the intron. Approximately 50% of the individuals we tested carried 3 tandem 31 bp repeats on one allele and 4 tandem repeats on the other allele (Fig. 1). The remainder of the individuals were homozygous for either one of the variants, in an approximately equal ratio. These sequence variants were observed in unrelated, unaffected individuals, in some occasions from different geographic regions, in both the homozygous and heterozygous states; therefore, we concluded that they are polymorphisms.

TABLE 4

Comparison of Exon-Intron Organization of the LAMC Genes

Exon	LAMC1	LAMC2	LAMC3
E1	418 + UTR	79 + UTR	373 + UTR
E2	305		305
E3	131		131
E4	167		167
E5	189	189	189
E6	118	136	118
E7	99	99	99
E8	137	137	137
E9	123	123	180
E10	190	190	194
E11	113	113	114
E12	222	219	118
E13	189	183	189
E14	246	246	246
E15	154		154
E16	143	143	143
E17	179		179
E18	157	157	143
E19	206	206	206
E20	80	80	76
E21	138	156	135
E22	145	145	149
E23	150	153	150
E24	115	115	103
E25	200	200	200
E26	159	159	150
E27	100	100	100
E28	254 + UTR	105 + UTR	248 + UTR

Note. The table compares the exon structure of the genes encoding the laminin γ chains. The exons are listed according to the exon numbering of LAMC1, the table shows the exon sizes in basepairs.

DISCUSSION

In this study, we present the complete exon-intron organization of the human laminin γ3 gene. The overall structure of LAMC3 is most similar to the LAMC1 gene. The homologues of LAMC1/LAMC3 exons 2–4, 15, and 17 are not present in LAMC2, but throughout the rest of the gene, LAMC2 also shows a significant homology to LAMC1 and LAMC3 (Table 4, Fig. 2).

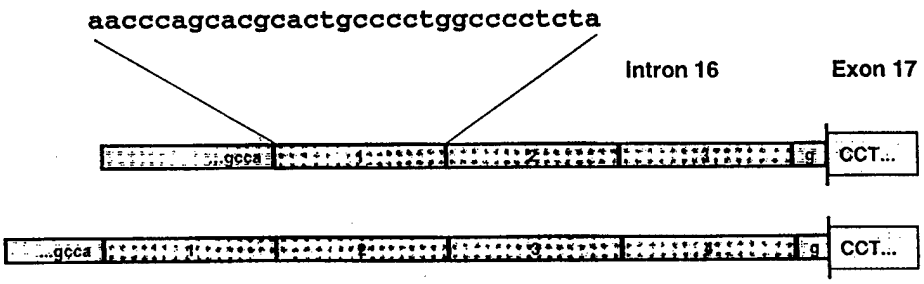

FIG. 1. Tandem repeat polymorphism in intron 16 of the LAMC3 gene. The figure shows the 31-bp tandem intronic sequence polymorphism. The blocks with plain shading demonstrate the 31-bp sequence, repeated three or four times. The blocks with horizontal pattern represent the remaining portion of intron 16 and the blocks with dotted pattern correspond to exon 17.

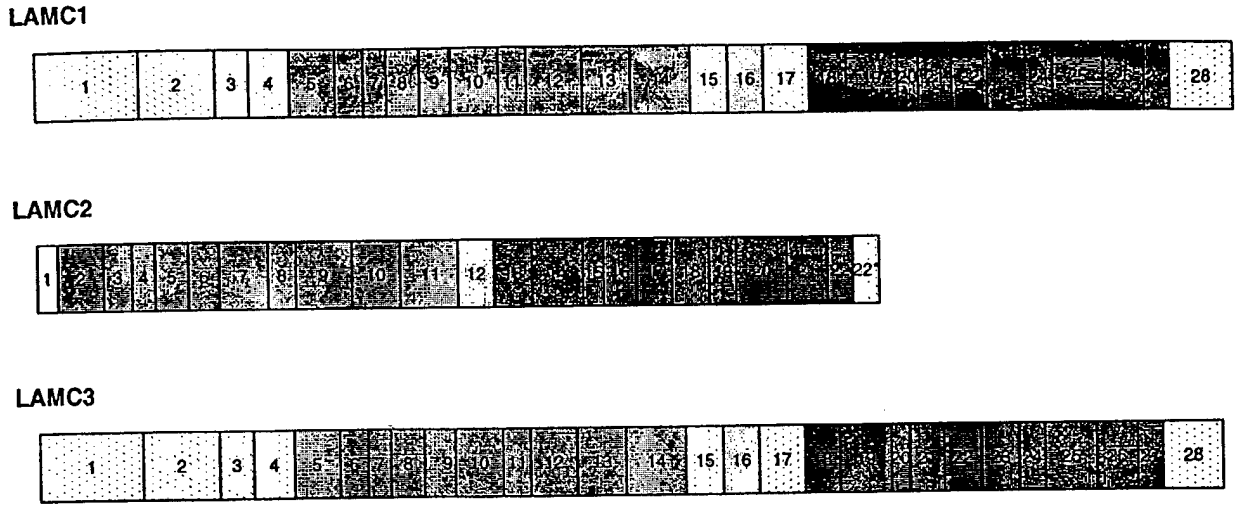

FIG. 2. Comparison of the exon arrangement of the LAMC genes. The diagram demonstrates the arrangement of exons with identical or highly similar size (plain shading). Exons showing significant size difference are marked with dotted pattern. Groups of corresponding exons marked with the same shading in the 3 genes. Note the high resemblance between LAMC1 and LAMC3.

Despite the strong structural homology, the LAMC3 gene demonstrates an expression pattern different from the LAMC1 and LAMC2 genes (4–6). Previous expression studies localized laminin γ3 in several human tissues. In particular, the expression of LAMC3 in ciliated epithelial apical surfaces such as in the retina, male and female reproductive tract and lung and expression in peripheral nerve endings, raised several possible candidate human disorders for mutations in this gene.

One candidate disease would appear to be deafness with vestibular dysfunction, based on both mapping data (7) and functional considerations (8). A mouse disorder known as whirler deafness, which presents with deafness and vestibular dysfunction in the adult mouse and manifest as head-bobbing and circling behavior, is a likely mouse model for similar human disorders. The syndrome thought to be a result of defects in the membranous labyrinth and the organ of Corti. In both of these organs, ciliated cells and nerve structures are critical for proper function. LAMC3 could be a valid candidate gene for this disorder, as its product, the γ3 chain, is expressed in both the sensory structures of the ear and the nerves innervating them (Brunken and Burgeson, unpublished observation). The possible role of LAMC3 in the whirler mouse is further supported by linkage analysis data. The whirler phenotype shows a recessive pattern of inheritance in mouse. Mapping positioned the whirler region on mouse chromosome 4 between the aminolevulinate dehydratase (Lv) and hexabrachion (Hxb) genes (7). Based on the chromosomal location of the human homologues of these genes (ALAD and HXB), the syntenic region in human was predicted to be 9q32–34, which corresponds to the position of LAMC3 (9q31–34) according to our radiation hybrid mapping results, as well as previously reported FISH data (Koch *et al.*, 1999).

Other diseases in which LAMC3 mutation may play a role can be selected on the basis of either chromosomal localization or predicted pathomechanism. Walker-Warburg syndrome (OMIM:236670), a recessively inherited disorder characterized by markedly disorganized cytoarchitecture, lack of lamination, numerous glial heterotopias, and encephalocele, in some cases is mapped to 9q31 (9). Fukuyama muscular distrophy (OMIM: 253800), characterized by cerebral and cerebellar micropolygyria, fibroglial proliferation of the leptomeninges, hydrocephalus, focal interhemispheric fusion, and hypoplasia of the corticospinal tracts is also mapped to 9q31 (10). The clinical characterization of these conditions is difficult due to the complexity of symptoms, therefore, the existence of endophenotypes is a strong possibility and may help to refine diagnosis in both diseases. Therefore, even though retrotransposon insertion in the fukutin gene has been reported to underlie about 85% of all Fukuyama cases (11), due to the suggestive linkage data and the expression of LAMC3 in the brain which is the primary focus of pathology in both cases, the pathogenetic role of LAMC3 mutations in the remaining cases of Fukuyama disease and Walker-Warburg syndrome should be investigated.

In this study, we present a mutation screening strategy based on PCR and heteroduplex analysis, that enables us to screen for mutations in candidate diseases. We tested our screening strategy on genomic DNA samples from unrelated healthy individuals and identified several sequence variants. Most of these sequence variants do not result in an amino acid substi-

tution, and all have been found in unrelated, clinically unaffected individuals. The previously published expression data demonstrate that LAMC3 is widely expressed; therefore, we predict that any significant impairment of the protein function could be related to clinical abnormalities.

Currently, laminin mutations have been shown to underlie junctional epidermolysis bullosa (12). Animal studies also provided insights into possible pathogenetic roles of different laminin molecules (13, 14), but due to the widespread expression of laminins deleterious mutations in most of the laminin molecules may not allow the development of a viable embryo, therefore those mutations would never lead to a disease, which could explain the relatively small number of human disorders successfully associated with mutations in the laminin genes. This study should facilitate the rapid identification of LAMC3 mutations in a candidate human disorder.

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Stem Cells in the Epidermis

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Key Words

Epidermis · Stem cells · Follicle bulge · Markers, stem cells · Niche, stem cells · Plasticity

Abstract

The epidermis consists of three actively proliferating units, the interfollicular epidermis, the hair follicle, and the sebaceous gland. Stem cells in the epidermis have the capacity to produce all three of these units. The fate of the epidermal stem cells and some of their progeny can be altered, dependent on the environment in which they reside and the genes they express. In this review, we describe the major experiments that have contributed to the understanding of the epidermal stem cells and the control of their fate.

Classical Approaches to Analysis of Stem Cells

Cells in the epidermis are constantly being lost, through the sloughing off of squames, the hair cycle, and the production of sebum. For this to occur, by definition, there must be a population of cells in the epidermis able to replace these lost cells. In other tissues, stem cells can produce differentiated progeny to compensate for the loss of differentiated cells. The evidence is strong that this is also the case in the epidermis. The mouse epidermis, when either severely injured or irradiated, is eventually reconstructed, presumably through a protected stem cell population [1–3]. These stem cells, which divide infrequently, but can effectively produce limitless progeny, give rise to more frequently dividing cells that can produce far lower numbers of progeny, known as transit-amplifying (TA) cells. It is these cells that divide to give rise to terminally differentiated progeny. Based on these properties, stem cells will incorporate a label during DNA

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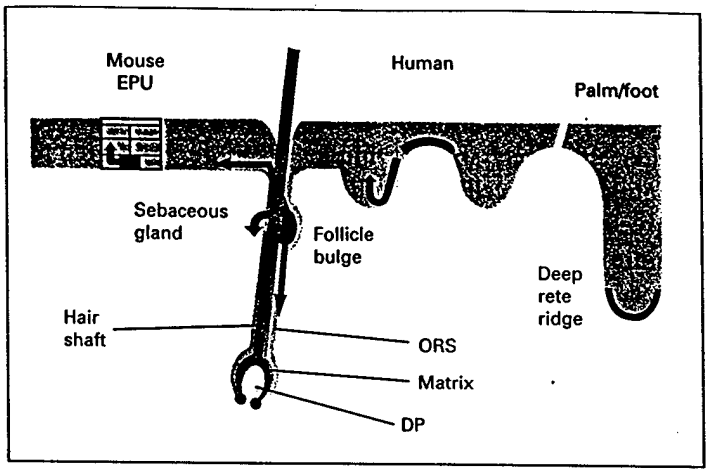

Fig. 1. A diagrammatic representation of the locations of cells of high proliferative potential in the epidermis. The red regions show the areas in which these cells are thought to reside. The blue arrows show known or inferred movement and differentiation of the stem cells. Note the red region at the base of the hair follicle. Clonogenic cells were observed in this region by Rochat et al. [16]. EPU = Epidermal proliferative unit; ORS = outer root sheath; DP = dermal papilla.

synthesis, such as tritiated thymidine or bromodeoxyuridine, and after a chase period of at least 4 weeks retain the label, whereas the labeled TA cells should have been lost through terminal differentiation. Studies of epidermal stem cells in the mouse revolve around the concept of the label-retaining cell. Small numbers of these are found in the basal layer of the mouse interfollicular epidermis [4]. In the mouse, the interfollicular epidermis is arranged in such a way that each squame overlies a column of epidermal cells. Single label-retaining cells are at the base of each of these columns, known as the epidermal proliferative unit (EPU; fig. 1), with the TA cells surrounding the label-retaining cell [5]. Cells destined for terminal differentiation leave the basal layer at the edges of the EPU. Label-retaining cells may additionally be the target of cutaneous mutagens. Preneoplastic or initiated cells must persist in the epidermis to promote the formation of papillomas and carcinomas. Argyris [6] showed that repeatedly abraded skin, containing only hair follicles, could produce tumors, suggesting that stem cells may reside in the hair follicles. Recently, Morris et al. [7], showed that the interfollicular epidermis may also contain initiated cells, consistent with the EPU hypothesis.

In humans, assays for label-retaining cells are not possible. However, culture of human keratinocytes using the techniques established by Rheinwald and Green [8] has long been used to produce epidermal sheets for grafting onto burns [9]. The fact that Gallico et al. [9] demonstrated that the epidermis produced from these grafts is permanent suggests that cells with stem-like properties were retained in some form for the period the cells were in culture. When keratinocytes are grown at clonal density, that is, when a single cell gives rise to a colony of keratinocytes, then only 20–40% of the cells plated typically give rise to colonies [10], with differences being observed with body site of the primary keratinocytes and age of the donor [8]. These colonies have properties that are based on the keratinocyte that gave rise to them and can roughly be divided into two types: large and proliferative colonies, typically containing thousands of cells, and small (normally 40 cells or less) colonies, where all the cells are expressing markers of terminal differentiation [10]. It is assumed that the founders of the large prolifera-

tive colonies are stem cells and that the founders of the small, terminally differentiated colonies are TA cells. However, one essential characteristic of stem cells is their ability to divide effectively without limit. Therefore, the ability of the cells of a proliferative colony to give rise to further proliferative colonies is a more stringent assay for stem cells [11]. Three colony types are observed using this approach: a founder that produces little or no secondary colonies, where all the colonies expressing terminal differentiation markers are referred to as paraclones; founders producing no terminal colonies are referred to as holoclones, and founders with intermediate characteristics are referred to as meroclones. All three of these founders can produce proliferative colonies (and hence are stem cells according to the criteria mentioned above), but have differing proliferative potential [11]. Even the least proliferative founders, the paraclones, have more proliferative potential than the TA cells, as described above. Both the assays described are informative in terms of retrospectively defining cells with high proliferative potential, and although more robust, the holoclone/paraclone/meroclone assay is considerably more complex to perform than proliferative versus abortive colonies. In any case, keratinocytes in vitro differentiate through holoclones to paraclones before becoming TA cells destined for terminal differentiation.

Thus, epidermal stem cells in mouse and human must be tracked down using different approaches, with label retention being the method of choice in the mouse and in vitro keratinocyte cell culture in the human. However, the questions remain the same in both species. Where are the stem cells? What genes do they express? To what cell types do they give rise? Novel applications of the concepts described above are bringing us closer to answering these questions and further understanding the epidermal stem cell.

Where Are the Stem Cells?

It has long been thought that any stem cell must reside in a protected milieu, widely referred to as a 'niche', safe from the damaging (and mutating) influences of the environment [12, 13]. Until recently, apart from the label-retaining cells of the EPU, no other epidermal stem cell locations were known. There are two obvious protected regions of the epidermis: the first is the hair follicle, and the second are the deep folds, known as the rete ridges in human epidermis [14] (fig. 1). Cotsarelis et al. [15] found label-retaining cells in a very specific part of the hair follicle, named the bulge (fig. 1). Human hair follicles exhibit a large number of proliferative founders in the equivalent region [16], providing the first indirect evidence that label-retaining cells have a high proliferative potential in vitro. Recently, direct evidence of this has been found in a study where FACS revealed that label-retaining cells in the bulge of mouse hair follicles are founders of high proliferative potential [17].

The positions of stem cells in the mouse are the hair follicle bulge and the bottom of the EPU. In the human hair follicle bulge, there are also cells of high proliferative potential. So, where are the interfollicular stem cells in humans (fig. 1)? It is believed that there must be interfollicular stem cells, since the major sources of keratinocytes, the neonatal foreskin as well as other body sites such as palm and soles, have no hair follicles. Jones et al. [18] revealed that there is a variation in expression of β1 integrin in frozen sections of epidermis, with the areas of basal epidermis closest to the surface of the skin expressing the highest levels of the integrin and the areas furthest away, the rete ridges, expressing the lowest. Maybe paradoxically, cells from these regions of the epidermis showed high and low proliferative colony-forming ability, respectively. Wholemount staining of human epi-

Table 1. Exp

Gene
β1 Integrin
α6 Integrin
β-Catenin
E-Cadherin
c-myc
Keratin 15
Keratin 19
p63
Delta1

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Table 1. Expression patterns of markers of stem cells and determinants of stem cell fate

Gene	Outer root sheath	Inner root sheath	Follicle bulge	Human basal epidermis	Mouse basal epidermis	Other locations
β1 Integrin	+	–	+	high expression closest to skin surface	+	
α6 Integrin	+	–	+	all of basal layer	+	
β-Catenin	+	+	–	high expression in rete ridges	+	bulb of hair follicle
E-Cadherin	+	+	–	high expression in rete ridges	+	hair matrix
c-myc	–	–	+	all of basal layer	–	bulb of hair follicle
Keratin 15	-	-	+	punctate staining throughout basal layer	ND	bulb of hair follicle
Keratin 19	+	–	+	deep rete ridges in palm/foot	–	Merkel cells
p63	+	–	+	individual cells throughout basal layer	+	corneal stem and young TA cells
Delta1	–	–	–	high expression close to skin surface	ND	dermal condensation of developing hair follicle

Expression in a tissue is shown by +, lack of expression in a tissue is shown by –; ND = not determined. Expression in the human epidermis is described, while 'other locations' describes other skin/epithelia-specific locations in which these genes are expressed.

dermis shows the organization of the epidermis into regions of high β1 integrin expression surrounded by regions of low β1 integrin expression [19], where expression of markers of terminal differentiation, for example keratin 10, can be found. There is evidence that this organization is brought about in part both by differences in keratinocyte motility and the interactions between the Notch genes and one of their ligands, Delta1, both of which are classical 'boundary-forming' genes in many organisms [20].

Markers of the Stem Cell

To isolate more pure populations of epidermal stem cells, it is necessary to identify better molecular markers to differentiate them from their progeny. To date, using classical approaches as well as differential gene expression analysis and two-dimensional gel electrophoresis [21], it has been difficult to find markers of the stem cell. Extracellular matrix receptors, such as integrins, and cytoskeletal components, such as cytokeratins, are attractive markers of stem cells, with most of the effort in this direction being focused on the β1 integrin [8] and the α6 integrin [22] in the interfollicular epidermis and keratins 15 and 19 in the hair follicle [23, 24]. Keratin 15 shows no expression in the basal epidermis [25]; however, keratin 19 is additionally expressed in the β1 integrin high region [26]. The distribution of β1 integrin has already been discussed, but α6 integrin may also be used as a marker to sort stem cells from the basal population. Although α6 integrin is expressed at the same level throughout the basal layer of the epidermis, it is believed that making a cell suspension for FACS allows the exposure of more α6 integrin on the surface of

the keratinocytes. This allowsfor enrichment of stem cells by doubly sorting for α6 integrin high expressing and transferrin receptor low expressing cells [17]. Recently, the p53 family member p63 has become very attractive as a candidate marker of stem cells in both human and mouse. Homozygous mice null for p63 show an absence of all stratified epithelia, presumably due to a lack of stem cells [27, 28]. Human p63 is expressed in holoclones and meroclones, but not paraclones, in culture [29]. What is more interesting is the fact that stem cells in the corneal limbus, the position of which is well characterized, express high levels of p63 [29]. The one piece of the p63 puzzle missing is the distribution in human epidermis. Will the p63-positive cells correlate with a subset of either the β1 integrin high expressing cells or the α6 integrin high and transferrin receptor low expressing cells?

Of the markers described, Delta1 is expressed on the cell surface. Unfortunately Delta1 expression in epidermis is coincident with that of high levels of β1 integrin [20]. There is an increasing need for better cell surface markers, so that the isolation of viable epidermal stem cells can be achieved, allowing insight into their gene expression and other properties.

Potentiality and Plasticity of the Epidermal Stem Cell

Stem cells are responsible for the renewal of the hair follicle as well as the continued proliferation of the epidermis. Is a single cell capable of performing both functions, or is there a form of lineage restriction that prevents a keratinocyte from assuming one or the other fate? In the mouse, the bulge cells repopulate the epidermis after damage [1], and very recently, it has been shown that in the neonate, label-retaining cells that can still divide move out of the hair follicle and into the interfollicular epidermis [30]. The reverse also appears to be true, with interfollicular keratinocytes in the adult rat footpad producing hair follicles in the presence of vibrissa dermal papilla cells [31], arguing for at least a bipotentiality of the epidermal stem cell. However, only by tracking the progeny of a stem cell can a definitive conclusion be made about what appendages in the skin it contributes to. Genetic labeling of keratinocytes, typically with alkaline phosphatase or beta galactosidase, and mixing with unlabeled keratinocytes reveals that not all structures in the epidermis are uniformly labeled. EPUs are clearly marked in the mouse [32, 33], but frequently not all the structures in the hair follicle are uniformly labeled [33, 34], suggesting a multiclonal origin of the hair follicle, with different programs of lineage restriction giving rise to the different structures.

Very recently, Oshima et al. [35] performed a convincing experiment providing evidence for the multipotentiality of adult epidermal stem cells. Replacing the bulge region of the mouse vibrissa follicle with a bulge from a mouse expressing beta galactosidase in all cells demonstrated that the bulge keratinocytes contribute to all the epidermal lineages. Colony-forming assays also allude to the movement of keratinocytes of high proliferative potential down the vibrissa follicle to the bulb region during a normal hair cycle. If the stem cells are moving, an obvious question is what prevents them from differentiating en route. As with hematopoietic stem cells and neural stem cells, it is probable that the epidermal stem cells need to be in a specialized microenvironment that allows them to properly proliferate and differentiate. Or, put more controversially, are keratinocytes only ever stem cells when they are located in the correct niche?

Just how important is the niche in controlling the fate of stem cells and their progeny?

The dermis clearly has an important role in determining the fate of epidermis. Dermal fibroblasts from palm and sole have the potential to induce trunk-derived keratinocytes in culture and grafts to produce the palmoplantar keratin 9 [36]. So the dermal niche can significantly alter the fate of the keratinocytes above it. However, it was widely regarded that adult stem cells are essentially unipotent, inasmuch as they can only produce the tissue from which they originate, be it skin, muscle, blood cells or the CNS. Presumably it would take extensive reprogramming to alter the fate of an adult stem cell enough to produce another tissue. Evidence that adult stem cells from tissues, such as blood, muscle, and CNS, can undergo reprogramming is being steadily accumulated. Stem cells in the CNS can adopt a hematopoietic fate in bone marrow repopulation assays [37]. Hematopoietic stem cells also seem to be able to adopt a muscle fate and vice versa [38, 39]. Stem cells from the muscle and bone marrow can be isolated on the basis of exclusion of the nuclear dye Hoechst 33258, due to high levels of a multiple drug-resistance-like gene [39, 40]. It has yet to be conclusively proven that the same technique could be used to isolate a population of pluripotent cells in the epidermis or that epidermal stem cells can give rise to other tissues. However, Ferraris et al. [41] have recently demonstrated that central corneal epithelium, which contains only proliferative TA cells and not stem cells, when combined with embryonic dermis, produces epidermis as well as appendages specific to body location, either hair follicles or sweat glands. Presumably this is the result of signals from the dermis reprogramming the determined corneal transit amplifying cells and allowing them to adopt a stem-cell-like fate, and is the first evidence of such developmental plasticity in keratinocytes [41].

The Future: New Approaches and New Concepts

The classical approaches described above are reaching the limit of what they can teach us about epidermal stem cells, and through the increasing body of knowledge, there has been a gradual change in the questions being addressed. In our opinion, there are at least three problems that need to be addressed. First, isolating a pure epidermal stem cell population, most likely without the intermediate step of cell culture, will be central in elucidating the expressed genome of the stem cell. Differences in gene expression (if any) compared to its progeny will focus attention on the genes important in maintaining the stem cell phenotype. The function of these genes in the epidermis is likely to be determined through transgenic mice. However, it is probable that some if not all of these genes will be essential for the development of many organs, thus presenting a second challenge. To circumvent potential problems, inducible tissue-specific knockouts, using a combination of Cre-Lox technology and hormone-inducible gene fusions [42], are currently being used with some of the potential candidate genes (table 1); β1 integrin ablation in the basal epidermis causes severe epidermal, basement membrane, and follicular defects [43, 44]. Induced expression of the proto-oncogene c-myc in the basal epidermis increases the amounts of sebocyte differentiation as compared with follicular differentiation, while continuous expression of c-myc under the control of the keratin 14 promoter reduces the number of label-retaining cells in the epidermis [45, 46]. Intriguingly, ablation of β-catenin, a gene genetically upstream of c-myc, prevents cycling of hair follicles by forcing epidermal stem cells into an interfollicular fate [47]. Finally, steps towards elucidating what signals in the dermal and epidermal

components of the niche regulate stem cell fate and potentially regulate any developmental plasticity will have to be undertaken. There is the potential for epidermal stem cells to be not only useful in treating cutaneous diseases, but as an easily obtained source of pluripotent stem cells that could repopulate a range of tissues.

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